Interactions between Heterotypic Stressors and Corticosterone Reveal Integrative Mechanisms for Controlling Corticotropin-Releasing Hormone Gene Expression in the Rat Paraventricular Nucleus

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Although the convergence of neural and humoral afferent information onto paraventricular neuroendocrine corticotropin-releasing hormone (CRH) neurons is a major determinant for adaptive stress responses, the underlying integrative mechanisms are poorly understood. To dissect the relative contributions made by neural afferents and corticosterone to these processes, we determined how the concurrent application of two heterotypic physiological stressors, chronic dehydration (produced by drinking hypertonic saline) and sustained hypovolemia (produced by subcutaneous injections of polyethylene glycol), is interpreted by the synthetic and secretory activity of CRH neurons using *in situ* hybridization and plasma ACTH measurements. These two stressors are encoded by relatively simple, distinct, and well defined sets of neural afferents to CRH neurons. Both increase plasma corticosterone, but they have opposing actions on *CRH* gene expression when applied separately. In the first experiment, we showed that chronic dehy-

dration suppresses *CRH* gene transcription after hypovolemia, but not the preproenkephalin and *c-fos* mRNA responses or ACTH secretion. In the second, we showed that negative feedback actions of corticosterone do not suppress *CRH* gene activation after hypovolemia, but instead determine the prestress lower limit of a range within which the CRH gene then responds. Collectively, these data show that at least two processes are integrated to control how the *CRH* gene responds to multiple stimuli. First, the presence of corticosterone, which although permissive for appropriately activating the CRH gene during hypovolemia, does not mediate the suppressed gene response. Second, neural afferent-driven processes that encode dehydration play a central role in suppressing CRH activation.

Key words: corticosterone; stress; adaptation; integration; neuropeptides; afferent control

The medial parvocellular paraventricular nucleus (PVHmp) is the focus for the neural control of the hypothalamo-pituitary-adrenal axis. A subset of PVHmp neurons synthesize the corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) released into hypophyseal portal blood, which in turn control the synthesis and release of ACTH from corticotropes. CRH neurons receive diverse sets of neural and humoral inputs, particularly corticosterone, which are integrated by the PVHmp to provide coordinated and adaptive hormone release.

To maintain the viable pool of releasable CRH within terminals of neuroendocrine neurons, stimuli that increase ACTH release are generally accompanied by increased *CRH* gene transcription and synthesis of the bioactive peptide. Thus, many stressors increase CRH heteronuclear (hn) RNA (the primary gene transcript) and CRH mRNA levels (Kovács and Sawchenko, 1996a,b; Ma et al., 1997a; Tanimura et al., 1998). However, it is unclear how the stimulus/synthesis/secretion sequence for CRH is controlled by the neural processes that integrate inputs to the

PVHmp and ultimately determine adaptive neuroendocrine responses to stress.

We have investigated this integrative process by determining how neuropeptide genes respond to an acute stressor that normally stimulates their expression (sustained hypovolemia) when it is presented together with a heterotypic chronic stressor that depresses *CRH* gene expression (cellular dehydration; DE). Because these stressors mediate their opposing effects on gene expression using simple, restricted, and well defined sets of neural afferents (Kovács and Sawchenko, 1993; Pacák and Palkovits, 2001; Watts, 2001), how they interact when presented concurrently should provide insights about integrative processes in a way not possible using more complex stressors.

Sustained hypovolemia, as evoked by injections of polyethylene glycol (PEG), produces intensity-dependent increases in ACTH and corticosterone secretion lasting at least 5 hr (Stricker et al., 1979; Tanimura et al., 1998). Increased ACTH secretagogue release is followed by pronounced activation of CRH and other neuropeptide genes (Watts and Sanchez-Watts, 1995b; Tanimura et al., 1998). However, *AVP* gene expression is not increased by hypovolemia (Tanimura et al., 1998). The response of neuroendocrine CRH neurons to the DE evoked by increasing plasma osmolality is quite different. DE maintained for up to 5 d increases plasma corticosterone concentrations and slowly decreases levels of CRH biosynthesis (Young, 1986; Dohanics et al., 1990a; Watts, 1992; Watts et al., 1995; Kay-Nishiyama and Watts,

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1999). DE also reduces the ACTH secretory response to some stressors (Dohanics et al., 1990b; Aguilera et al., 1993).

Corticosterone is generally regarded as a simple negative feedback signal that regulates *CRH* gene expression in the basal state and during stress, although the underlying mechanisms have proved remarkably elusive (Watts, 1996; Kovács et al., 2000). Corticosterone controls the onset, magnitude, and duration of the neuroendocrine *CRH* gene response to hypovolemia (Tanimura and Watts, 1998, 2000), whereas circulating corticosterone is a prerequisite for DE-associated reductions in *CRH* mRNA (Watts and Sanchez-Watts, 1995b). To determine what role corticosterone might play in the integrative control of *CRH* gene expression, we also measured in adrenalectomized (ADX) rats how different corticosterone concentrations affected the magnitude of the *CRH* mRNA response to PEG injections.

MATERIALS AND METHODS

Animals and treatments. Adult male Sprague Dawley rats (225–250 gm body weight at the beginning of the experiment) were maintained on a 12 hr light/dark photoperiod (lights on at 6:00 A.M.) with *ad libitum* access to water and rat chow and were allowed at least 5 d of acclimation to the animal quarters. At this time (day 0) one batch of rats was divided initially into two groups: (1) animals maintained on water and (2) animals given 2.5% saline (w/v) to drink for 3 d. This exposure to DE was chosen because this was the minimum length of time required to reduce *CRH* mRNA in the PVHmp (Watts et al., 1995).

On the morning of day 3 (6:30–7:30 A.M.), food and water or saline were removed from both groups and rats were briefly anesthetized with halothane. Rats in both groups were then given subcutaneous injections of either 5 ml of 40% PEG (MW 8000; Sigma, St. Louis, MO) dissolved in 0.9% saline or 5 ml 0.9% saline and left undisturbed until they were killed 5 hr later. Injections were given through a small incision in the midline of the back using a 10 cm, 21 gm blunt needle directed toward the neck. The incision was sealed using a wound clip to prevent leakage.

Rats from each of the treatment groups were then killed in one of two ways. Some were deeply anesthetized by intraperitoneal injection of tribromoethanol, and a single 1–1.5 ml blood sample was taken from the external jugular vein into a heparinized syringe for hematocrit and osmolality measurements. Animals were then perfused through the ascending aorta with a brief saline rinse followed by 500 ml of ice-cold 4% paraformaldehyde solution in 0.1 M borate buffer, pH 9.5. After perfusion, the brain from each animal was removed and postfixed overnight at 4°C in the fixative containing 12% sucrose (w/v). Brains were frozen in hexanes cooled in powdered dry ice and immediately stored at –70°C until sectioning at a later date. Eight series of one-in-eight, 15- μ m-thick frontal sections were cut through the rostral hypothalamus and saved in ice-cold potassium PBS, pH 7.4, containing 0.25% paraformaldehyde; sections were handled and stored as described previously (Watts and Sanchez-Watts, 1995a). Adjacent sections were saved for thionin staining.

Because the anesthesia required for perfusion precluded the measurement of plasma ACTH and corticosterone, a second batch of animals treated in the same manner as the first was killed by rapid decapitation. Trunk blood was collected in two cooled vials, coated with either EDTA-saline for ACTH assay or heparin-saline for corticosterone assay. Small samples from the heparinized blood were used for hematocrit and osmolality measurements. An index of the plasma volume deficit was derived from the hematocrit using the following relationship: $[(\text{hematocrit}_{\text{PEG}} - \text{hematocrit}_{\text{mean control}})/(\text{hematocrit}_{\text{mean control}})] \times 100$. Thymuses were removed from animals after decapitation, blotted dry, and weighed. Because thymus weights are negatively correlated with $\log_{10}$ plasma corticosterone concentrations in a manner that is dependent only on glucocorticoid receptors (Dallman et al., 1987; Watts and Sanchez-Watts, 1995c), they provide an additional dependent variable for determining the bioactivity of circulating corticosterone concentrations. This measurement is particularly useful for assessing the long-term efficacy of circulating corticosterone during extended periods of DE, because characterizing the varying dynamics of plasma corticosterone concentrations is not easily determined without frequent sequential blood samples (Watts, 1992, 2000).

In a separate experiment designed to determine the effects of corticosterone on the *CRH* mRNA response to sustained hypovolemia, rats

maintained throughout with water were bilaterally ADX under halothane anesthesia using flank incisions. At this time they were given a subcutaneous capsule containing either 25, 50, or 100 mg of corticosterone (Innovative Research of America, Sarasota, FL). The time course of corticosterone release from these capsules together with their effects on *CRH* mRNA levels and thymus weights have been characterized in previous publications (Swanson and Simmons, 1989; Watts and Sanchez-Watts, 1995c; Tanimura and Watts, 1998). Six days later, rats were given a subcutaneous injection of either 40% PEG or vehicle (four to six animals per corticosterone capsule group), as described above. After 5 hr, rats were rapidly anesthetized with tribromoethanol and then perfused as just described. A 6 d period was chosen in this experiment to ensure that mRNA levels had stabilized after adrenalectomy and capsule implantation (Swanson and Simmons, 1989). Thymuses were removed after perfusion and weighed. Brains were processed as described above to measure relative *CRH* mRNA levels in the PVH. Determinations of both thymus weights and *CRH* mRNA were made in the majority but not all animals.

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Southern California.

In situ hybridization. Sections were hybridized with ³⁵S-UTP-labeled cRNA probes transcribed from cDNA sequences encoding RNAs for parts of the following genes: preproCRH (700 bp), preproenkephalin (ppENK; 935 bp), a 536 bp *PvuII* fragment complementary to the sequence within the single *CRH* intron, and rat *c-fos* (2.1 kbp). Although AVP is colocalized with *CRH* in the PVHmp (Whitnall, 1993) and plays a significant role in regulating the ACTH response to some stressors (Antoni, 1993; Kovács et al., 2000), we did not measure AVP hnRNA or mRNA levels in this study, because sustained hypovolemia does not increase parvicellular AVP gene expression (Tanimura et al., 1998). Furthermore, DE significantly increases circulating corticosterone, which in turn strongly inhibits the AVP gene and its response to hypovolemia (Kovács et al., 2000; Tanimura and Watts, 2000). It seemed reasonable to assume that combining these stressors would be ineffective at activating this gene.

cRNA probes were synthesized using the Promega (Madison, WI) Gemini kit and the appropriate RNA polymerase. The characterization of all probes has been reported previously (Watts, 1992; Watts and Sanchez-Watts, 1995a; Tanimura et al., 1998). *In situ* hybridization with the ³⁵S-labeled cRNA probes was performed as described previously (Watts and Sanchez-Watts, 1995a; Kovács and Sawchenko, 1996a) with posthybridization modifications to the *CRH* hnRNA hybridization as follows. After the RNase incubation at 37°C and room temperature washes from 4 to 0.1 $\times$ SSC, slides were incubated at 70°C for 30 min with slight agitation every 10 min. Sections were exposed to Microvision C x-ray film (Diagnostic Imaging Inc., Mira Loma, CA) for appropriate exposure periods (2–42 d), dipped in nuclear track emulsion (Kodak NTB-2, diluted 1:1 with distilled water; Kodak, Rochester, NY), exposed for 5–42 d, developed, and counterstained with thionin.

Semiquantitation of ³⁵S-UTP-cRNA hybridization. Mean gray levels of the RNA hybridization signals in the Nissl-defined subdivisions of the PVH were measured from images on Microvision C x-ray film as described previously (Watts and Sanchez-Watts, 1995a; Watts et al., 1995). Parcellation of the PVH was determined using the scheme and nomenclature of Swanson (1998). We have previously demonstrated the linearity of the *in situ* hybridization signal response on the x-ray film and our detection system (Tanimura et al., 1998).

Radioimmunoassays. Plasma corticosterone and ACTH concentrations were measured in duplicate unextracted samples as described previously (Tanimura et al., 1998) using a [¹²⁵I]corticosterone or [¹²⁵I]ACTH double antibody radioimmunoassay supplied in kit form (ICN Biochemicals, Costa Mesa, CA). The lower sensitivity limits were 12.5 ng/ml and 15 pg/ml, and the intra-assay coefficient of variation was <8.1 and 9.6% for corticosterone and ACTH, respectively. All samples were measured in single assays.

Statistical analysis. The significance of differences in osmolalities, hematocrits, and RNA hybridizations was determined across treatment groups using one-way ANOVA, followed by Dunnett's two-tailed *post hoc* test; values from animals given water to drink and injections of isotonic saline were used as controls. The effects of saline or PEG injections on thymus weights or *CRH* mRNA levels in ADX animals with corticosterone implants were determined by analysis of covariance. $p < 0.05$ was regarded as being statistically significant. All statistical analyses were performed using Excel (Mac version 5.0; Microsoft, Seattle, WA) and Systat (Mac version 5.2).

Table 1. Effect of sustained hypovolemia on mean $\pm$ SEM thymus weights, plasma osmolalities, and hematocrits from animals given either water (W) or 2.5% hypertonic saline (HS) to drink for 3 d, followed by a 5 ml subcutaneous injection of 0.9% saline (Veh) or 40% PEG, and killed 5 hr later

	Treatment			
	W/Veh	HS/Veh	W/PEG	HS/PEG
Thymus weights (mg)	560 $\pm$ 27	372 $\pm$ 36*	525 $\pm$ 21	323 $\pm$ 30*
% change from control	0.0 $\pm$ 4.9	–33.5 $\pm$ 6.4	–6.3 $\pm$ 3.8	–42.3 $\pm$ 6.3
Number of animals per group	5	6	6	7
Osmolality (mOsm/kg H ₂ O)	287 $\pm$ 2	322 $\pm$ 4*	309 $\pm$ 3*	325 $\pm$ 3*
% change from control	0.0 $\pm$ 0.7	12.4 $\pm$ 1.3	7.6 $\pm$ 1.1	13.2 $\pm$ 0.9
Number of animals per group	4	7	6	6
Hematocrit (%)	47.1 $\pm$ 0.8	54.9 $\pm$ 0.6*	60.6 $\pm$ 1.1*	63.5 $\pm$ 0.6*†
Relative plasma volume deficit (%)	0.0 $\pm$ 0.8	12.5 $\pm$ 0.9	24.8 $\pm$ 1.3	25.8 $\pm$ 0.7
Number of animals per group	6	7	6	6

Relative plasma deficit was calculated using the mean W/Veh value used as a control for all groups.

* $p < 0.01$ or greater versus W/Veh animals.

† $p < 0.01$ or greater versus appropriate non-PEG-injected controls.

RESULTS

Effects of dehydration on thymus weights, plasma osmolality, and hematocrit

Table 1 shows that drinking 2.5% saline for 3 d led to increased plasma osmolalities in all animals. Animals drinking water and injected with 40% PEG also showed increased plasma osmolality, although the size of this increase was significantly smaller than that seen after hypertonic saline ingestion and was most likely a result of the increased plasma protein concentrations resulting from hypovolemia (Stricker and Jalowiec, 1970). Plasma sodium levels are reduced by this treatment (Watts and Sanchez-Watts, 1995a). Three days of drinking 2.5% saline led to a significant reduction in thymus weights that was not further affected by PEG injection (Table 1).

In animals drinking water, PEG injections were followed 5 hr later by a significant reduction in plasma volume as reflected by increased hematocrits (Table 1). Three days of ingestion of hypertonic saline was followed by a significant reduction in plasma volume in animals injected with isotonic saline, but this was then reduced much further in the DE animals injected with PEG (Table 1).

Drinking hypertonic saline had no effect on the mean plasma ACTH concentration in animals injected with saline vehicle but did significantly increase mean plasma corticosterone concentrations (Fig. 1). These data are consistent with the existence of

Figure 1. The effect of drinking either water (W) or hypertonic saline for 3 d (HS) on the mean $\pm$ SEM plasma concentrations of ACTH (A) and corticosterone (B) measured 5 hr after either subcutaneous vehicle (Veh) or PEG (P) injections. *ns*, Not significant.

Figure 2. The effect of drinking either water (W) or hypertonic saline for 3 d (HS) on the mean $\pm$ SEM CRH mRNA (A) and CRH hnRNA (B) levels in the medial parvocellular part of the hypothalamic paraventricular nucleus measured 5 hr after either subcutaneous vehicle (Veh) or PEG (P) injections.

Figure 3. Photomicrographs of the hypothalamic paraventricular nucleus [approximately level 26 of Swanson (1998)] showing the effects of drinking either water or hypertonic saline for 3 d (*HS*) on the response 5 hr later to either subcutaneous vehicle (*Veh*) or PEG (*P*) injections of CRH mRNA, CRH hnRNA, ppENK mRNA, and *c-fos* mRNA as detected by *in situ* hybridization. The *top* row shows adjacent thionin-stained sections (*Nissl*). Each *column* shows adjacent sections from one animal chosen as representative of each treatment.

non-ACTH-dependent mechanisms for increasing plasma corticosterone in DE animals. We have suggested previously that changes in corticosterone catabolism contribute to this effect (Watts, 2000), perhaps mediated by reduced hepatic clearance rates (Woodward et al., 1991). Mean plasma ACTH and corticosterone concentrations were robustly increased in all animals injected with PEG; drinking water or hypertonic saline had no significant effect on the mean concentration attained by either hormone 5 hr after PEG injections.

Effects of dehydration on the response of CRH hnRNA and mRNA levels to hypovolemia

For animals drinking water, CRH mRNA levels in the PVHmp were significantly increased 5 hr after PEG injections (Figs. 2*A*, 3). Figures 2*A* and 3 also show that 3 d of drinking 2.5% hypertonic saline significantly reduced the levels of CRH mRNA

in the PVHmp and completely suppressed its response to PEG at this time.

CRH hnRNA levels were significantly increased above control levels 5 hr after PEG injections in animals provided with drinking water (Figs. 2*B*, 3). Although these hnRNA responses to PEG injections were still elevated above control values in animals given hypertonic saline to drink, they were significantly attenuated when compared with responses present in animals drinking water. The reason why CRH mRNA levels were unaffected by hypovolemia in dehydrated animals while hnRNA levels increased (albeit in a significantly blunted manner) is unclear. However, it may be related to the technical difficulty of measuring small changes in the relatively large cytoplasmic mRNA pool. Other groups have reported a similar discrepancy in response to some stressors (Kovács and Sawchenko, 1996).

Figure 4. The effect of drinking either water (W) or hypertonic saline for 3 d (HS) on the mean + SEM level of preproenkephalin mRNA (A) and *c-fos* mRNA (B) in the medial parvocellular part of the hypothalamic paraventricular nucleus and *c-fos* mRNA in the posterior magnocellular part of the hypothalamic paraventricular nucleus (C) measured 5 hr after either subcutaneous vehicle (Veh) or PEG (P) injections.

Figure 5. Thymus weights (A) and CRH mRNA levels in the medial parvocellular part of the hypothalamic paraventricular nucleus (B) were significantly correlated to the $\log_{10}$ plasma corticosterone concentration in ADX animals given a subcutaneous pellet containing various doses of corticosterone and injected with either 0.9% saline vehicle (○; dashed line) or 40% PEG (●; solid line). PEG injections did not effect either the slope or the Y intercept of the relationship between corticosterone and thymus weights but did significantly increase the Y intercept of the relationship between corticosterone and CRH mRNA levels. See Results for levels of statistical significance.

Effects of dehydration on the response of *c-fos* and ppENK mRNA levels to hypovolemia

Levels of both ppENK (Figs. 3, 4A) and *c-fos* (Figs. 3, 4B) mRNAs in the PVHmp were unaffected by drinking hypertonic saline, but both were significantly increased 5 hr after PEG injections. The magnitude of the increase in ppENK and *c-fos* mRNA levels after hypovolemia was unaffected by drinking hypertonic saline (Figs. 3, 4A,B).

Figures 3 and 4C show that *c-fos* mRNA was significantly increased in the posterior magnocellular part of the PVH (PVHpm) in animals drinking hypertonic saline and injected with saline vehicle. However, hypovolemia also increased *c-fos* mRNA levels in the PVHpm in both groups of animals. These data support the idea that DE does not globally suppress gene responses to hypovolemia in the neuroendocrine hypothalamus. In fact, in contrast to parvocellular CRH neurons, the effects of DE and sustained hypovolemia appear to be integrated in an additive

manner in magnocellular neuroendocrine neurons, at least as far as *c-fos* gene expression is concerned.

Effects of corticosterone on the response of thymus weights and CRH mRNA levels to hypovolemia in euhydrated ADX animals

Plasma corticosterone concentrations in capsule-implanted ADX animals ranged from 62 to 508 ng/ml (Fig. 5A,B). Figure 5A shows that thymus weights were significantly correlated to the $\log_{10}$ plasma corticosterone concentration in animals injected with vehicle ($r^2 = 0.838$; $F = 72.52$; $p < 0.0001$; $n = 16$) or PEG ($r^2 = 0.912$; $F = 133.96$; $p < 0.0001$; $n = 15$). Analysis of covariance showed that PEG did not have a significant effect on corticosterone concentrations ($p = 0.17$) in animals used for the thymus weight determinations. Furthermore, injection with PEG had no significant effect on either the slope ($p = 0.76$) or the Y intercept ($p = 0.14$) of the relationship between plasma corticosterone

concentration and thymus weight. Although a direct comparison of absolute thymus weights was not possible because of the different methods used for killing (perfusion vs direct decapitation), comparing the relative reduction found in animals after 3 d of DE (~38%; Table 1) with those in corticosterone-replaced animals (Fig. 5A) suggested that mean plasma corticosterone concentrations of intact animals over the 3 period of dehydration were equivalent to that produced by ~150 ng/ml corticosterone in the hydrated corticosterone-replaced animals.

Figure 5B shows that there was a significant negative correlation between CRH mRNA levels in the PVHmp and the log₁₀ concentration of corticosterone in both vehicle-injected animals ($r^2 = 0.519$; $F = 16.20$; $p < 0.002$; $n = 17$) and PEG-injected animals ($r^2 = 0.506$; $F = 14.38$; $p < 0.002$; $n = 16$). Analysis of covariance showed that PEG injection did not have a significant effect on corticosterone concentrations ($p = 0.562$) in animals used for CRH mRNA determinations. Like thymus weights, the slopes of the regression lines between plasma corticosterone concentration and CRH mRNA levels were not significantly different between animals injected with vehicle or PEG ($p = 0.08$). However, the Y intercept was significantly different between those animals injected with vehicle and those with PEG ($p < 0.001$).

DISCUSSION

We have shown previously that sustained hypovolemia activates *CRH* gene transcription in PVHmp neurons within 2 hr of the onset of hypovolemia and is maintained for the duration of the stress (Tanimura et al., 1998). Chronic DE however, downregulates CRH synthesis in these same neurons and requires at least 3 d to manifest its effects on *CRH* gene expression (Young, 1986; Dohanics et al., 1990; Watts, 1992; Aguilera et al., 1993; Watts et al., 1995; Kay-Nishiyama and Watts, 1999). We now show that the consequences of DE also restrain *CRH* gene activation in response to sustained hypovolemia to the extent that CRH mRNA is no longer significantly increased after PEG injections. The fact that increased CRH hnRNA levels are also strongly attenuated in these circumstances suggests that DE inhibits, at least in part, the effects of the PVHmp afferent mechanisms used by sustained hypovolemia either before or during *CRH* gene transcription. Because the *CRH* gene response to hypovolemia is suppressed, whereas those of the *ppENK* and *c-fos* genes remain unaffected means that this inhibition is not directed nonspecifically at PVHmp neurons. This conclusion is also supported by the fact that the magnitude of the ACTH response to hypovolemia in DE animals is indistinguishable at 5 hr from control animals, and shows that neuroendocrine CRH neurons can still release ACTH secretagogues in a stimulus-dependent manner into the hypophyseal vasculature. Whether these observations mean that gene expression and peptide release are each driven by separate afferent mechanisms, or whether DE preferentially suppresses intracellular mechanisms that control *CRH* gene expression is not yet clear. However, our previous observations showing that the onsets of peptide release and gene activation have different stress-intensity thresholds (Tanimura et al., 1998) are consistent with the notion that there is at least some degree of separation between the mechanisms that activate secretion and gene transcription.

It is reasonable to consider that the opposing actions of DE and sustained hypovolemia on *CRH* gene expression are, like those of other stressors, mediated by two processes. First, the two sets of neural afferents respectively encode the sensory information gen-

erated by DE and hypovolemia and converge on the PVH. This convergence may occur directly at CRH neurons in the PVH, or more distally in sets of neurons that then project to the PVH. Second, corticosterone-dependent mechanisms involve actions on CRH neurons themselves as well as more indirect effects mediated by afferents to the PVHmp (Herman and Cullinan, 1997; Kovács and Sawchenko, 2000). With regard to neural afferents, the reduced *CRH* gene expression that follows DE requires cells in the vascular organ of the lamina terminalis but not ascending projections from the hindbrain (Kovács and Sawchenko, 1993). DE also increases neuropeptide gene expression in a subset of lateral hypothalamic neurons that project to the PVH, which may also modulate CRH neurons (Champagne et al., 1998; Watts et al., 1999). In contrast, hypovolemia activates CRH gene expression in the PVHmp using a different set of neural afferents. These include angiotensinergic inputs from the subfornical organ and ascending catecholaminergic projections from the hindbrain that encode various types of hemodynamic sensory information (Chan and Sawchenko, 1994; Pacák and Palkovits, 2001).

The second important regulator of CRH synthesis in the PVHmp is corticosterone. Many workers have demonstrated its robust negative feedback relationship with CRH mRNA levels in the PVHmp (for review, see Watts, 1996). In unstressed rats, this is seen as a negative logarithmic correlation between plasma corticosterone concentrations and CRH mRNA levels (Watts and Sanchez-Watts, 1995c). At least part of the mechanism used by corticosterone to reduce CRH mRNA levels involves an action on *CRH* gene transcription; CRH hnRNA levels are significantly increased in ADX animals when compared with intact rats or ADX rats with subnormal corticosterone replacement (Tanimura and Watts, 1998, 2000; Kovács et al., 1998, 2000). However, a variety of temporal and molecular data suggest that unlike its control of the AVP gene (Burke et al., 1997), corticosterone does not reduce CRH mRNA by direct receptor-mediated inhibitory actions on *CRH* gene transcription (Ma et al., 1997b; Reichardt et al., 1998; Tanimura et al., 1998). Presumably, other nongenomic actions that target the processes preceding transcriptional activation are important for the negative feedback actions of corticosterone (Rosen et al., 1992; Guardiola-Diaz et al., 1996).

Corticosterone is required for the actions on *CRH* gene expression of both stressors used in this experiment. Thus, DE-dependent reductions of CRH mRNA do not occur in ADX animals (Watts and Sanchez-Watts, 1996b), whereas the level of circulating corticosterone present before the stress determines how the *CRH* gene responds to sustained hypovolemia (Tanimura and Watts, 1998, 2000). It is important to note that for hypovolemia, the dose of corticosterone that normalizes *CRH* gene responses of ADX rats is well below the maximum corticosterone values seen during either mild stress or normal circadian variations (Tanimura and Watts, 1998, 2000). This observation is similar to that reported by Dallman et al. (1987), who showed that corticosterone replacement at levels around the circadian mean, 30–60 ng/ml, will normalize a variety of components in the pituitary–adrenocortical system.

Although these negative feedback actions of corticosterone are consistent with the idea that the elevated corticosterone concentrations seen during DE (Watts, 1992; Watts et al., 1999) might be responsible for suppressing *CRH* gene responses to sustained hypovolemia, our data now suggest that this is unlikely, because high levels of corticosterone did not suppress the *CRH* gene response to PEG in non-DE animals. Thus, we show that during hypovolemia there is still a significant negative relationship be-

tween circulating corticosterone concentrations and the CRH mRNA levels attained during the stress, the slope of which is not significantly different from that in nonstressed animals. Critically however, the regression line is significantly shifted to the right for the relationship between corticosterone and CRH mRNA levels after PEG, but not, as one might expect, thymus weights. These data show that if circulating corticosterone is held between the circadian mean and ~300 ng/ml before the stress occurs, it does not inhibit the ability of the CRH gene to respond to PEG, a finding consistent with our previous study (Tanimura and Watts, 1998). The principal effect of corticosterone on the CRH gene response to hypovolemia, therefore, is to control prestress mRNA levels; it has much less of an effect on the magnitude of the gene response. Thus, our data show that although corticosterone is required, the chronically elevated plasma concentrations seen in DE animals cannot be responsible for inhibiting the CRH mRNA response to sustained hypovolemia, which leaves an afferent-dependent mechanism as the most parsimonious explanation.

Collectively, our observations show that part of the integrative control of CRH gene expression in PVHmp neurons requires interactions between the different sets of neural afferents that encode the diverse types of sensory information generated by physiological stimuli. In other words, how one set of neural afferents affects CRH gene expression at any one time is dependent on the status of others. We suggest that the reason the CRH gene response to hypovolemia is greatly attenuated by DE is because it changes the state of DE-sensitive PVHmp afferents (Kovács and Sawchenko, 1993; Watts, 2001) in such a way as to reduce the efficacy of the afferents that normally enable sustained hypovolemia to activate the CRH gene.

In this model, the way corticosterone controls the gene response to a particular stressor is dependent on which set of afferents is activated at a particular time. Thus, when circulating corticosterone is at or above the circadian mean, it negatively regulates the lower level of an operating range within which the CRH gene might respond to stressors. Whether corticosterone influences the magnitude of the gene response is determined by the nature of the stressor and the afferent set it uses. With hypovolemia, which is encoded by one set of afferents, we show that corticosterone does not inhibit the CRH gene response. However, corticosterone has a much greater inhibitory action on the CRH gene response to more complex stressors, such as ether inhalation, that are encoded by different afferents (Kovács et al., 2000). Our interpretation regarding steroid-sensitive and -insensitive gene activation is reminiscent of a similar dichotomy for ACTH secretory responses to various stressors (Keller-Wood and Dallman, 1984; Thiruvikraman and Plotsky, 1993), the basis of which is reportedly related to differential activation of PVHmp afferent sets (Thiruvikraman et al., 2000; Pacák and Palkovits, 2001).

Finally, these integrative mechanisms also target neuropeptide genes that are coexpressed with CRH. This is clear for the AVP gene, where corticosterone has a profound influence on the magnitude of its response to stress (Tanimura et al., 1998; Kovács and Sawchenko, 2000; Tanimura and Watts, 2000). However, corticosterone and DE have no detectable effect on the ppENK gene or its response to sustained hypovolemia (Watts, 1992; Watts and Sanchez-Watts, 1995c; Tanimura and Watts, 1998). In summary, this entire integrative process might be considered as a form of corticosterone-dependent switching that determines how the expression of peptide genes in neuroendocrine CRH neurons responds to differential activation of their afferent inputs.

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